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PRINCIPAL INVESTIGATOR: XiaoHe Yang, Ph.D.

CONTRACTING ORGANIZATION: University of Oklahoma Health Sciences Center
Oklahoma City, Oklahoma 73117-1213

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6. AUTHOR(S)
XiaoHe Yang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma Health Sciences Center
Oklahoma City, Oklahoma 73117-1213

E-Mail: xiaohe-yang@ouhsc.edu

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13. ABSTRACT *(Maximum 200 Words)* This project was proposed to study the correlation between caspase deficiency and breast cancer carcinogenesis and therapeutic resistance. In the last year of this grant, we examined the mRNA levels of 8 caspases in 20 breast cancer cell lines and the expression of caspases- 3 in 102 cases of breast cancer tissues. We also studied the role of caspase-3 in genistein induced apoptosis. In summary of the whole project, we have examined the expression profiles of the majority members of the caspase family in 20 breast cancer cell lines and performed functional screening of these cell lines treated with different apoptotic stimuli. Our results indicated that caspase deficiency/downregulation was frequently detected in breast cancer and suggested a correlation between caspase deficiency and breast carcinogenesis and therapeutic resistance. Using a caspase-3 specific model, we demonstrated that caspase-3 plays a critical role in the apoptosis of breast cancer cells. Deficiency of caspase-3 significantly impaired cellular response to various apoptotic stimuli, which contributed to therapeutic resistance. Sensitization of caspase-3 deficient cells by transfection of the caspase-3 gene suggests that caspase-3 gene therapy could be used to enhance therapeutic responses of refractory breast cancers.

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INTRODUCTION

Apoptosis (programmed cell death) is a fundamental process involved in homeostasis and the biochemical responses to different anti-tumor therapies. Aberrant expression of other apoptotic regulators, such as Bax-α and p53, has been associated with carcinogenesis and therapeutic resistance of breast cancer. Based on the fact that caspases are a group of proteases that mediate apoptotic execution, and that caspase 3 is deficient in MCF-7 breast cancer cells, we hypothesized that caspase deficiency/downregulation could be common in breast cancers, which would contribute to breast carcinogenesis and therapeutic resistance. This project was proposed to study the significance of caspase deficiency in breast cancer carcinogenesis and resistance. The specific aims are:

1. To determine the incidence and pattern of caspase deficiencies in breast cancer via screening for specific caspase expression in breast cancer derived cell lines, explant cultures and snap frozen human breast cancer tissue.
2. Correlation of caspase data (obtained in aim 1) with apoptosis induction using immune modulators (TNF-α/Fas-ligand, GrB/Ad), chemotherapeutic agents and radiation on breast cancer derived cell lines and explant cultures.
3. Define the biologic role of specific caspase deficiencies via reconstitution of deficient caspases and comparative studies to define apoptosis induction in response to immune modulators, chemo- and radiation therapy.

This is the final report of the funded project. I will summarize the results from previous annual reports and discuss in detail about the results derived in the last year (2003-2004). The presentation will be in the order that was proposed in the original grant application.

BODY

Task 1. Detection of caspase expression in commonly used breast cancer cell lines and tissues. The objective of this task was to examine whether the expression of caspases is modified among different breast cancers. As proposed, we performed the following experiments.

1. We collected and expanded about twenty breast cancer cell lines for later examination of caspase expression. We also tried to establish primary cell lines from breast cancer tissues as described in a previous report (year 2000). However, despite the great effort, due to the outgrowth of fibroblasts from breast cancer derived explants, the primary cell culture appeared not practical without intensive characterization.

2. We then examined the protein levels of nine caspases (Caspases 1-4, 6-10) in nineteen breast cancer cell lines. The results indicated that the protein levels of individual caspase varied from cell line to cell line. Variations in caspases 3, 6, 8 and 10 among different cell line were more frequent. Downregulation of one or more caspases was detected in most breast cancer cell lines. These results provided fundamental support of our hypothesis and established the basis for later experiments. Details were described and discussed in previous annual reports (year 2000 and 2001).

3. Examination of the mRNA levels of caspases in breast cancer cell lines (Accomplished in the last year).

To examine whether changes in caspase expression also occurs at the mRNA level, we extracted total RNA from 19 breast cancer cell lines and examined the mRNA levels of different caspases by RT-PCR (This was the equivalent to the northern blotting experiments proposed in the original application.). Two sets of multiplex PCR kits from Intergen, Co. (NY) were used for the detection. After reverse transcription, the cDNA samples derived from each cell line were amplified to detect multiple caspases in one reaction. Primers in set 1 multiplex were designed to detect caspases 2, 4, 6, 7, and 10. Set 2 multiplex detected caspases 3, 5, 8, 9 and Apoptosis Protease activating factor-1 (Apaf-1). Both sets used GAPDH as a housekeeping gene control. As shown in Figure 1 and 2 of the appendix, the mRNA levels of caspases also varied significantly from cell line to cell line. Relative mRNA levels of different caspases were analyzed using a semi-quantitative image analysis software (Fig. 3 of the Appendix).

Results from these experiments indicated that regulation of caspase mRNA levels was involved in the regulation of overall caspase expression. Variations in mRNA levels were universal. It is interesting that downregulation of caspase 10 mRNA levels was the most common one. Analysis

of the mRNA levels in context with corresponding protein levels of a given caspase in the same cell line indicated that mRNA and protein levels were consistent in most caspases. For example, both mRNA and protein levels of caspase-10 in SK-BR-3 and MDA-MB-415 cells were lower than that of the other cell lines. In general, downregulation of caspases 3, 6, 8, and especially 10 mRNA levels was more common, similar to what we observed at the protein levels. However, different patterns of mRNA and protein levels of a given caspase in some of the cell lines were also observed, suggesting the existence of posttranslational regulation, which is common when the cells undergo spontaneous or induced apoptosis. Although the huge information of 8-9 caspases in about 20 cells lines requires more detailed analysis, these results support our hypothesis that caspase deficiency/downregulation is common in breast cancers, which might contribute to tumor development and therapeutic resistance. What are the underlying mechanisms of caspase downregulation and whether the downregulated mRNA was associated with gene mutation should be investigated in future studies.

4. Detection of caspase expression in breast cancer tissues.

To support our in vitro finding, we examined the expression of caspases in breast cancer tissues. We first examined caspase 3 expression in 9 cases of snap-frozen breast cancer tissues. Three of the 9 samples displayed significant decrease of caspase-3 (reported in year 2000). We then examined caspase 3 expression in achieved paraffin embedded breast tissues using immunohistochemistry. We examined 30 cases in year 2000 and 102 cases in the last year. Based on the 132 cases we examined, 85 (64%) cases were caspase 3 positive. Forty seven (36%) cases were caspase-3 negative. In addition, results from our collaborator's lab (Dr. Kapil Mehta) (please see appendix C of year 2003 report) indicated that 75% of breast tumors lacked caspase-3 transcript (1). Although there is a discrepancy between the two laboratories, maybe due to different stringency control, the bottom line is that caspase-3 downregulation or deficiency is very common in breast cancers, which is the clinical evidence supporting our hypothesis. To find possible correlation between caspase expression and commonly used markers/factors in breast cancer diagnosis, we performed statistical analysis. Although we did not see a statistically significant correlation between caspase expression and node positivity and estrogen receptor (ER) status, conclusion should be drawn after the examination of more cases in the future.

Progression of testing the expression of other caspases in breast cancer tissues was not as good as we expected. Honestly, we have tried to establish immunohistochemistry conditions for testing caspases 6, 7, 8 and 9. However, due to antibody quality and problems associated with old achieved formalin fixed-paraffin embedded samples, the results were not consistent as caspase-3 in our preliminary. We'd rather test more sample than reporting less confident results. Although this funding cycle is ending, our studies on caspases expression will be continued.

In summary, the results from the breast cancer cell lines indicated that low or none detectable levels of caspases were present in a subgroup of breast cancer tissues/cell lines and breast cancer. Decrease or downregulation of caspase 3 levels may lead to the defect in apoptotic executions and contribute to breast cancer development and therapeutic resistance.

Task 2. Functional screening of breast cancer cell lines in response to immune modulators

The objective of this task was to perform functional screening of breast cancer cell lines in response to immune-modulators, such as granzyme B, TNF-α and/or Fas, and correlate their sensitivity with caspase expression profiles derived in task 1.

1. Apoptosis of breast cancer cell lines treated with TNF-α.

These experiments were performed in year 2 and 3 (please see the annual report of year 2001 and 2002). The results indicated that apoptotic responses to TNF-α were generally associated with caspase expression file, especially caspase-8, an apical caspase involved in the activation of death receptor mediated apoptosis. Among the cell lines tested, BT-20, MDA-MB-435s and MDA-MB436 cells, which express relatively higher levels of caspase-8, were very sensitive to TNF-α induced cell death. In contrast, HS-578T cells, which express lower levels of several caspases, including caspase-8, were relatively resistant to TNF-α. Interestingly, MDA-MB-231 cells, which were sensitive to other stimuli, was relatively resistant to TNF-α, maybe due to difference in death receptor activation.

2. Apoptotic responses of breast cancer cell lines treated with granzyme B induced apoptosis.

We performed functional screen of breast cancer cell lines treated with granzyme B in the third year (reported in year 2002). Granzyme B is a serine protease in the cytotoxic granules released by NK or CTL cells. Cell lines that displayed relative resistance to granzyme B were BT-474,

SK-BR-3, MDA-MB-361, ZR-75-1 and MDA-MB-415. Consistent with our previous report showing that granzyme B activates caspase cascade at multiple points upstream of effector caspases, it appeared that downregulation of one or more effector caspases (caspases 3, 6 and 7) might be associated with granzyme B induced apoptosis.

Task 3. Analysis of chemotherapeutic agents and radiation mediated killing in breast cancer cell lines

1. Caspase expression and cellular response to chemotherapeutic agent doxorubicin.

As reported in year 2001, functional screening of 19 breast cancer cell lines treated with chemotherapeutic agent doxorubicin indicated that MDA-MB-415, BT-483 and MDA-MB-435s, which had the downregulation of one or more caspases (results in the reports of year-2000 and 2001, Fig. 1. and 2 of appendix in this report), were relatively resistant to the treatment. MDA-MB-468 and MDA-MB-436 cells, which express relatively higher levels of most caspases, were very sensitive to doxorubicin. Interestingly, HS-578T cells, which were relatively resistant to TNF-α were sensitive to doxorubicin, suggesting that cellular response to different apoptotic stimuli may be different depending on the variation in the activating mechanism.

2. Caspase expression and cellular response to radiation treatment

Examination of the sensitivities breast cancer cell lines to radiation was reported in the year 2002. We assessed the cell death of 14 cell lines treated with radiation. MDA-MB-330, MDA-MB-134VI, MDA-MB-361 and ZR-75-30 cells, which had overall lower levels of caspases, were resistant to radiation. MDA-MB-231 and MDA-MB-468 cells, which have overall higher levels of caspases, were sensitive to radiation. A general correlation between caspases expression profiles and radiation sensitivity is suggested.

To summarize the results from functional screening of breast cancer cell lines treated with different apoptotic stimuli, we found a general correlation between caspase expression profile and apoptotic response and that the correlation might be stimulus dependent. For example, TNF-α induced apoptosis seemed to be associated with caspase-8 levels. Granzyme B induced apoptosis seemed to be associated with effector caspase levels. However, we also realized that these were descriptive experiments. The correlations observed in the screening experiments have

to be confirmed use a specific model. That is one of the reasons that we weighed more on task 4 using a caspase-3 specific model, which indeed yielded specific and significant outcomes.

Task 4. Reconstitution of deficient caspases in corresponding cell lines and analysis of the restoration of their sensitivity to immune modulators, chemotherapeutic agents and radiation induced apoptosis

The objective of this task was to study the specific role of caspase deficiency identified in breast cancers. Since caspase-3 was deficient in MCF-7 breast cancer cell lines, we reconstituted caspase-3 in these cells. Using control and caspase-3 reconstituted MCF-7 cells, we have performed in-depth studies on the role of caspase-3 in apoptotic responses in breast cancer cells, as summarized below:

1. Reconstitution of caspase-3 sensitize MCF-7 to chemotherapeutic agent. Please see the appendix D of year 2001 report.
2. Reconstitution of caspase-3 enhances radiation induced apoptosis. Please see the appendix B of year 2002. The significance was that we demonstrated the critical role of caspase-3 in radiation induced apoptosis and found that different apoptotic stimuli may have preferred death substrates.
3. Caspase-3 has feed back action on apical caspases and cytochrome c release (reported in year 2002). These results suggest that caspase-3 deficiency would have broader impact on apoptotic responses.
4. Preliminary studies on the interaction between caspase-3, p53, Bcl-2 or Fas in the apoptosis of breast cancer cells. We found that caspase-3 deficiency would impair DNA damage induced apoptosis even if the cells express wt p53. Overexpression of Bcl-2 had a negative selection of MCF-7/caspase-3 cells. DNA damage treatment seemed to upregulate Fas level in MCF-7/caspase-3 cells, as compared to the control MCF-7 cells. More work could be done following these preliminary data. Please see the reports of year 2002 and 2003 for detail.
5. Critical role of caspase-3 in soy isoflavone genistein induced apoptosis (this was accomplished last year).

Genistein is the major isoflavone (plant based phytochemical) found in soy products. A diet high in soy products has been associated with a low incidence of breast cancer in Asian

women (2). Results from our studies demonstrated that genistein/soy has a chemopreventive effect on mammary tumor development (3, 4). However, the mechanisms of soy/genistein-mediated anticancer activity remain unclear. Available reports suggest that genistein functions as a selective estrogen receptor modulator, a tyrosine kinase inhibitor, and a topoisomerase II inhibitor, which can regulate cell growth and induce apoptosis. MCF-7 and MDA-MB-231 cells were the two most often used cell lines, which represent genistein resistance and sensitive cell lines respectively. The sensitivity between the two cell lines is usually explained only by their estrogen receptor (ER) status, i.e., MDA-MB-231 cells were sensitive because they are ER negative, and MCF-7 cells are resistance because they are ER positive (2). Based on our studies on caspase-3 deficiency, we hypothesized that caspase-3 status also contribute to the difference between the two cell lines. As show in Fig. 4, appendix A, MDA-MB-231 cells were sensitive to genistein, as indicated by their EC50, which was consistent with previous report. Since caspase-3 status was also distinctive between the two cell lines (Fig. 5A, Appendix A), we tested whether caspase-3 reconstitution modify genistein sensitivity of MCF-7 cells. As shown in Fig. 6 of Appendix A, caspase-3 reconstitution rendered MCF-7 cells more sensitive to genistein. Increased PARP cleavage in genistein treated MCF-7/c cells, as compared to the control, suggest the sensitization was through apoptosis (Fig. 7).

These experiments are not only a test of another apoptotic inducer. Our results demonstrated that caspase-3 status/activity contributes to the difference in genistein sensitivities between the MCF-7 and MDA-MB-231 cells, which is traditionally explained only by ER status. This is of great significance in studying the mechanisms of genistein mediated anti-breast cancer activities.

In addition to caspase-3 reconstitution, we also reconstituted caspase-10 in MCF-7 cells, which resulted in the sensitization of MCF-7 to etoposide induced apoptosis. Please see the report of year 2003.

KEY RESEARCH ACCOMPLISHMENT

1. Examination of the protein levels of 9 caspases in 19 breast cancer cell lines.
2. Examination of the mRNA levels of 9 caspases and Apaf-1 in 19 breast cancer cell lines (in the last year).

3. Immunohistochemistry examination of caspase-3 in 132 cases of breast caner tissues (102 cases were done in the last year).
4. Functional screening of 19 breast cancer cell lines treated with doxorubicin
5. Functional screening of 19 breast cancer cell lines treated with TNF-a
6. Functional screening of 19 breast cancer cell lines treated with Granzyme B.
7. Functional screening of 14 breast cancer cell lines to radiation.
8. Reconstitution of caspase-3 sensitization of MCF-7 cells to chemotherapeutic agent induced apoptosis.
9. The role of caspase-3 in radiation induced apoptosis
10. The role of caspase-3 in genistein mediated anti-cancer activities (done in the last year).
11. Feedback action of caspase-3 in TNF-α and doxorubicin induced apoptosis
12. Preliminary studies of the interactions between caspase-3 and p53, bcl-2, or Fas.
13. Reconstitution of caspase-3 mediated sensitization of MCF-7 cells to etoposide.

REPORTABLE OUTCOMES

A. Publications:

1. Yang XH, Sladek TL, Liu X, Butler BR, Froelich CJ, Thor AD. Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. Cancer Res. 2001, 61(1):348-54.
2. Devarajan E, Sahin AA, Chen JS, Krishnamurthy RR, Aggarwal N, Brun AM, Sapino A, Zhang F, Sharma D, Yang XH, Tora AD, Mehta K. Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene. 2002, 21(57):8843-51.

B. Meeting abstracts

1. Yang XH, Butler BR, Sladek TL, Khandekar J, Thor AD: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to chemotherapeutic agent induced apoptosis. 22nd Annual San Antonio Breast Cancer Symposium, San Antonio, Texas, December 8-11, 1999. *Breast Cancer Res Treat* 1999; 57:93.
2. Yang XH, Ellefson B, Thor A: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to radiation induced apoptosis. 91st Annual Meeting, AACR, San Francisco, California, April 1-5, 2000. *Proc Annu Meet Am Assoc Cancer Res* 2000; 41:A991.

3. Yang XH, Liu XS, Yang SH, Khandekar JD, Froelich CJ, and Thor AD, Feedback action of caspase 3 on apical factors, Molecular targets and cancer therapeutics, AACR-NCI-EORTC International Conference, Miami, Florida, Oct. 2001
4. Yang XH, Sladek T, Liu X, Yang S, Ellefson B, khandekar J, Froelich C and Thor AD. Critical role of caspase 3 in MCF-7 breast cancer cells in response to different anti-cancer agents, Era of Hope, DOD breast cancer research Program, Orlando, Florida, Sept. 2002

C. M.S. Student thesis (grant related project under PI's supervision)

The role of p53 in caspase activation during chemotherapy induced apoptosis in MCF-7 breast cancer cell line (by Alison Johnson), 2000

D. Manuscripts in preparation/submission

1. Yang XH, Ellefson B, Thor A: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to radiation induced apoptosis.
2. Yang XH, Liu XS, Yang SH, Khandekar JD, Froelich CJ, and Thor AD, Feedback action of caspase 3 on apical factors
3. Yang XH, Yang SH, Liu J, Liu X, Jones L, Edgerton S and Thor A, Gene expression profiles of caspases in breast cancer cells

Reprints will be sent to DOD after publication

Personnel receiving pay from the research effort

XiaoHe Yang, MD., Ph.D.

Ms. Wei Zheng

Ms. Joan Liu

CONCLUSIONS

Summary

This project was proposed to study the correlation between caspase deficiency and breast cancer carcinogenesis and therapeutic resistance. The experiments were designed to address questions in there aspects:

A. Detection of caspase expression in breast cancer cell lines and clinical samples.
B. Functional screening of breast cancer cell lines treated with different apoptotic stimuli to correlate caspase expression profile with their sensitivities to different therapeutic agents
C. Mechanistic study of the impact of caspase(s) deficiency on response to different stimuli.

In Aim 1, we examined gene expression of caspases in breast cancer cell lines and clinical samples. Our results indicated that caspase deficiency and/or downregulation, at both protein and mRNA levels, were frequently detected in breast cancer cell lines. Using immunohistochemistry, we also found that caspases 3 levels were very low or not detectable in about 36% of breast cancer specimens, suggesting that caspases deficiency/downregulation may be another rate-limiting factor that affects cellular responses to apoptotic stimuli in these breast cancers.

For Aim 2, we performed functional screening of breast cancer cell lines treated with TNF-α, doxorubicin and radiation. Overall, we observed a general correlation between low caspase levels and resistance to one or more apoptotic stimuli, such as low levels of caspases and resistance to TNF-α induced apoptosis in HT578 cells. However, due to the functional overlaps among different caspases and different initiation mechanisms behind different stimuli, it is necessary to confirm these correlations using a specific model system, like our caspases 3 model described below.

In Aim 3, we investigated the mechanisms behind caspase-3 deficiency-mediated apoptotic resistance. We reconstituted caspase 3 in MCF-7 cells by transfecting the cells with pBabe/puro retroviral vector encoding caspase 3 cDNA and characterized their response to several anti-cancer agents, including chemotherapy, radiation and tumor necrosis factor alpha (TNF-α) and phytoestrogen (genistein). Flow cytometry and MTT assays showed that caspase 3 expression rendered MCF-7 cells significantly more susceptible to these treatments. Apoptosis medicated by the reconstituted caspase 3 was demonstrated by increased DEVD cleavage activities, activation of effector caspases and cleavage of cellular death substrates. Using this caspase-3 specific cell line model, we found that caspase 7 is downstream of caspase 3 and that caspase 6 could be activated by caspase 3 and other apical caspases. Our results also showed that caspase 3 had feedback signals to apical caspases (8, 9 and 2), as demonstrated by both cell-free and whole cells systems. Enhanced mitochondrial depolarization and cytochrome c release was

also detected in caspase 3 expressing MCF-7 cells treated with radiation or doxorubicin. These results demonstrated that caspase 3 plays a central role in apoptosis induced by different anti-cancer agents, suggesting that caspase 3 deficiency might contribute to the therapeutic resistance of MCF-7 breast cancer cells. Since caspase-10 levels were also low in the MCF-7 cells, we also reconstituted MCF-7 cell with caspases 10, which resulted in the sensitization of these cells to etoposide.

Overall, this project was carried out as it was planned. Although detection of caspase expression in breast cancer tissues was not as sufficient as we expected, due to available reagent quality and the problems associated with old formalin fixed and paraffin embedded tissues, our results from about 9 caspases in 20 cell lines (MCF-7 and other 19 cell lines) with both mRNA and protein profiles and functional assays, plus caspase-3 data from clinical samples, strongly support the correlation between caspase deficiency/downregulation and apoptotic resistance. Using our caspase-3 specific cell line model, we have performed mechanistic studies to demonstrate the role of functional caspase-3, or the impact of caspase-3 deficiency on apoptosis of breast cancer cells.

"So What" Section

This project was proposed to address an important question related to breast cancer, which is whether caspase deficiency is involved in breast cancer development and therapeutic resistance. Indeed, collective results from this project support our hypothesis that caspase deficiency/downregulation would be frequently detected in breast cancers, which could contribute breast carcinogenesis and therapeutic resistance. Using a caspase-3 specific deficiency/reconstitution model, we demonstrated that caspase-3 deficiency had profound impact on therapeutic responsiveness. These results indicated that not only the activation process, but also the available levels of certain caspases, control cellular responsiveness to apoptotic stimuli, including therapeutic agents. Our findings suggest that caspase deficiency should be considered as a biomarker for therapeutically refractory breast cancers. It is suggested that caspase-delivering gene therapy could be used to sensitize therapeutic responses. More clinical related studies should be followed.

In addition to the support to our hypothesis, the results from this project also raised new questions for further studies on the critical role of caspases in breast carcinogenesis. Based on our results, examples of the questions to be addressed include:

1. What is the underlying molecular mechanism of caspase deficiency and downregulation? Available reports based on different tumors suggests that genetic mutation or epigenetic regulation (5-7), such as methylation could leads to defect in caspase expression and function (7). Along this line, we could perform microdissection and gene sequencing to identify the mutations and study the mechanisms that regulate caspase transcription and expression.

2. In our experiments, we found that activation of caspases 7, and partially in caspase 6, was caspase-3 dependent (8). This suggests that there is a mini-cascade activation process in the activation of effector caspases. While some reports support our finding, there was also report showing that caspase-7 activation is in parallel to caspase-3 (9). Therefore, it is necessary to clarify this issue. Confirmation caspase-7 as a down stream target of caspase-3 would suggest that caspase-3 deficiency/downregulation would impair caspas-7 activities even if caspas-7 expression is normal. Related experiments will be followed.

3. Although the list of proteins that can be cleaved by caspase activation is increasing, there are still numerous caspase substrates to be identified (10). Using the caspase-3 deficient and reconstitution model established in this study, we will identify novel caspase-3 substrates that may play a critical role in final cellular dysfunction.

4. In this project, we performed screening experiments on 9 caspases and focused on caspase-3 deficiency in later studies. Although we could not perform detailed studies on each of the caspases, the results provided clues to cell line selection and model establishment of other caspases for further studies.

In addition to above mentioned impact, our caspase-3 specific cell line mode system is a key accomplishment for carrying out caspase-3 related studies. Other than its application in our own studies, we have sent out numerous samples to other researchers who expressed interests in our cell line model. This amplified our efforts in studying the role of caspases in cancer development and therapeutic resistance.

Finally, as it was expected from the funding mechanism, which is a Career development award, this project brought me into breast cancer research field. I would like to thank the DOD Breast Cancer Research Program for the support.

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APPENDIX A

Fig. 1. mRNA levels of caspases 2, 4, 6, 7 and 10 in breast cancer cell lines. Total RNA was extracted from indicated cell lines. Three ug of total RNA was used for cDNA synthesis. mRNA levels of the selected caspases were amplified by PCR for 30 cycles using a multi-complex PCR kit (set-6) from Intergen Co. (NY) according to the manufacture's protocol. Selected boxes are examples of down-regulated caspase mRNA levels.

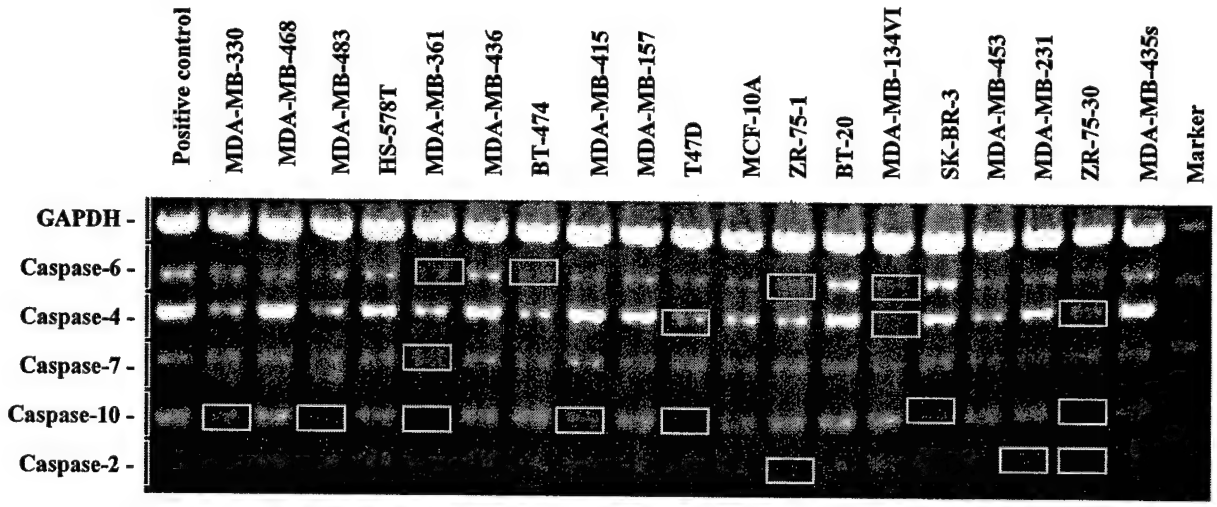
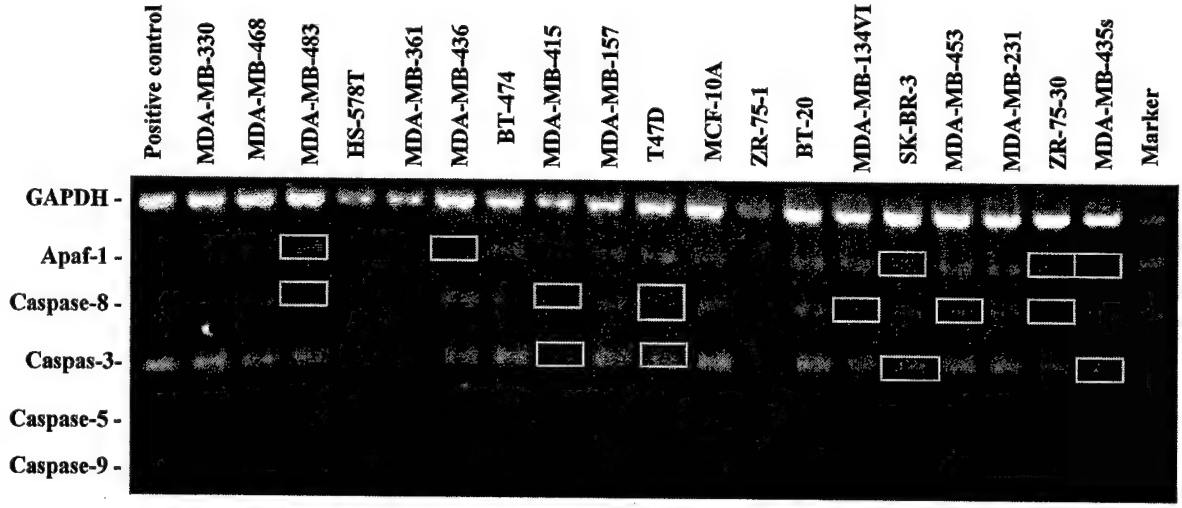
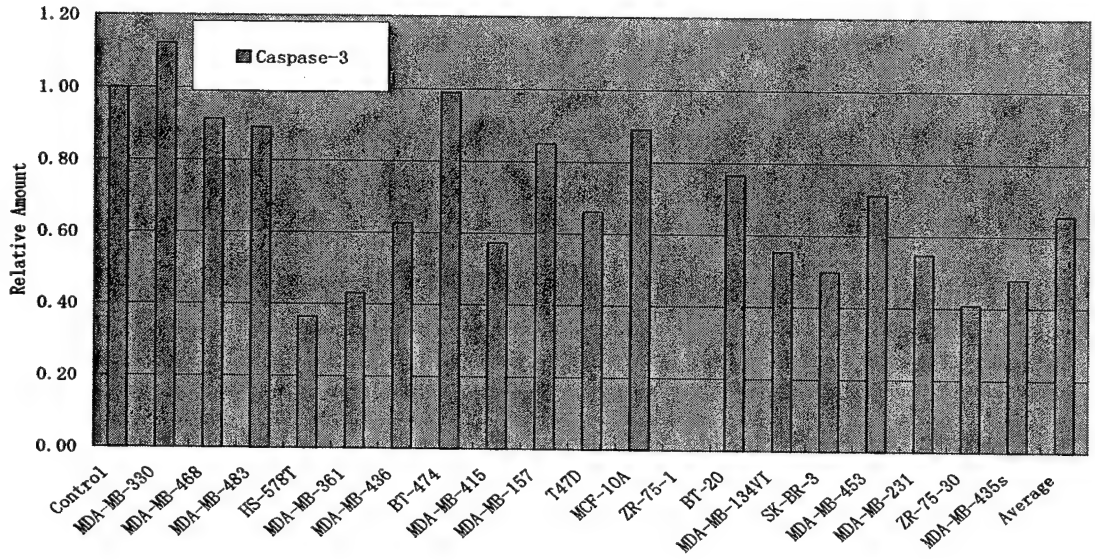


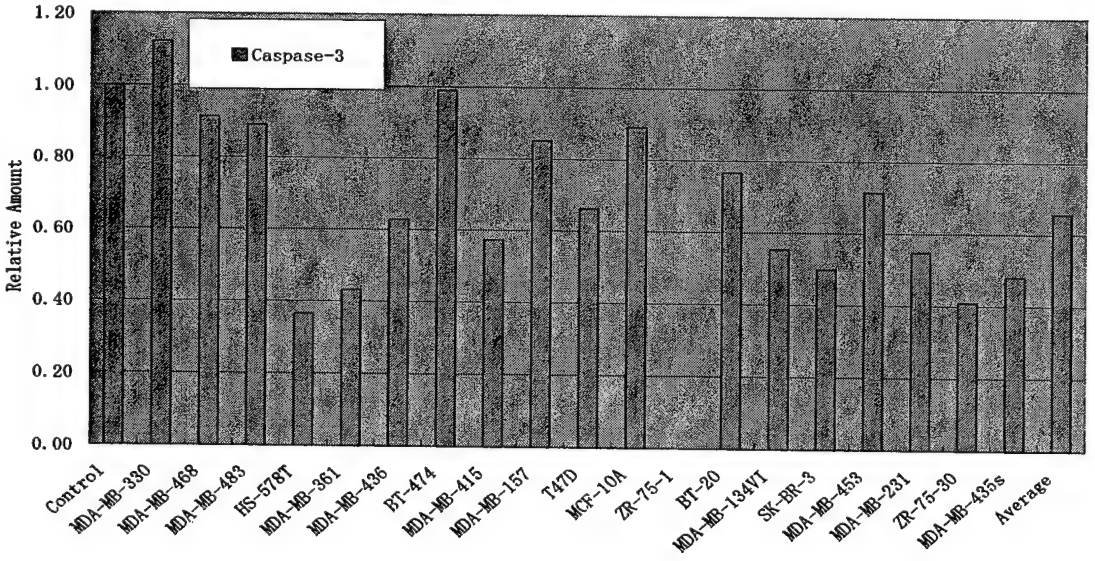
Fig. 2. mRNA levels of caspases 3, 5, 8, 9 and Apaf-1 in breast cancer cell lines. Total RNA was extracted from indicated cell lines. Three ug of total RNA was used for cDNA synthesis. mRNA levels of the selected caspases were amplified by PCR for 30 cycles using a multi-complex PCR kit (set-5) from Intergen Co. (NY) according to the manufacture's protocol. Selected boxes are examples of down-regulated caspase mRNA levels. It appears caspase 5 detection did not work well although the positive control was positive.

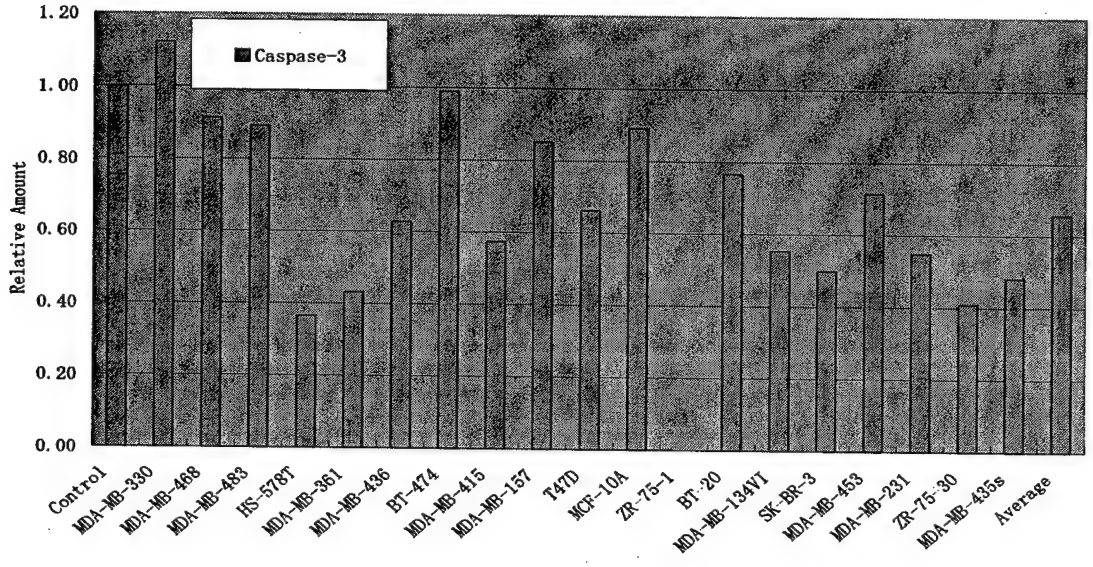


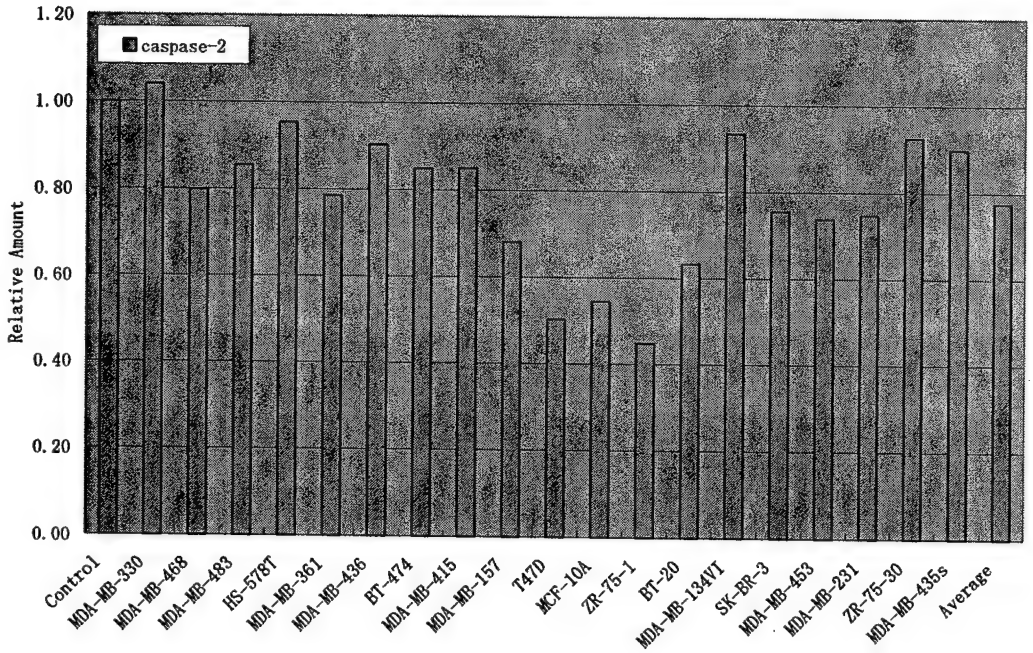
Appendix A

Figure 3: Expression profiles of caspases and Apaf-1 in breast cancer cell lines with semi-quantitative analysis. The relative amount of the PCR products corresponding to individual caspase in Figs. 1 and 2, which was acquired with a digital camera, was analyzed using the Eagle Eye Image analysis software. For the normalization, we first normalizes the signal of GAPDH of each cell line with the GAPDH signal of the positive control and generate a ration factor (GAPDH caspase/GAPDH control) that reflects the differences in loading among different samples. Signal of each caspase was further normalized with the ration factor (GAPDH caspase/GAPDH control). The signal of each caspase from the positive control lane is designated as 1. Relative amount of a certain caspase was calculated. Blank lane indicates that no adequate signal was acquired. For the same reason, results for caspases 9 and 5 was not presented.






Caspase-3
Relative Amount
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


caspase-2
Relative Amount
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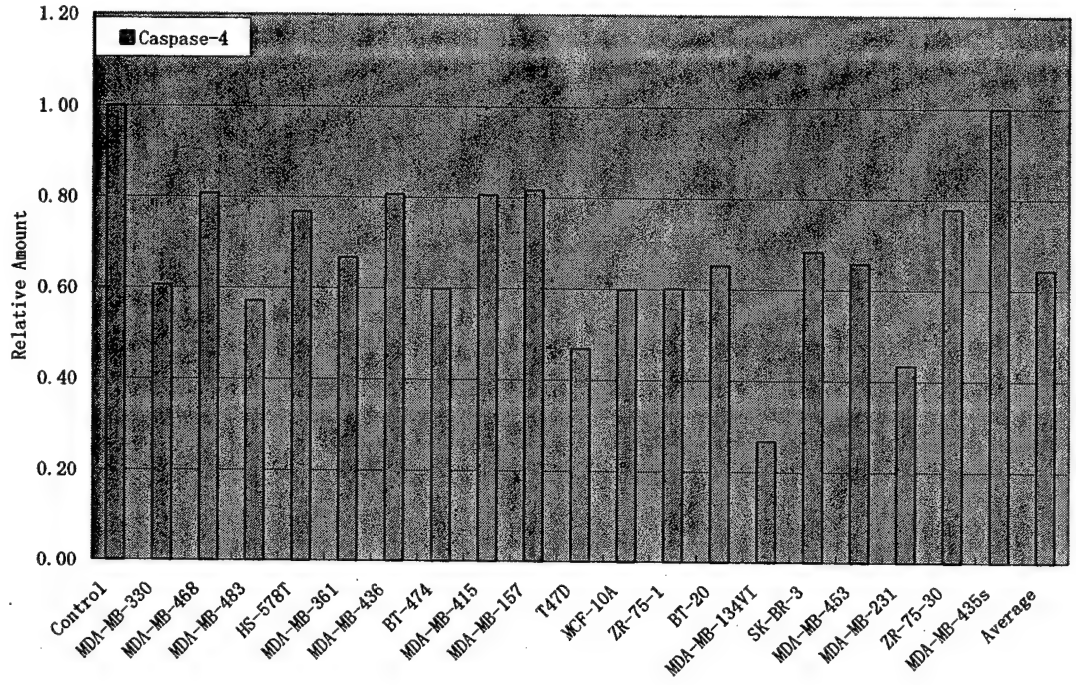
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


Caspase-4
Relative Amount
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1.00
0.80
0.60
0.40
0.20
0.00
Control
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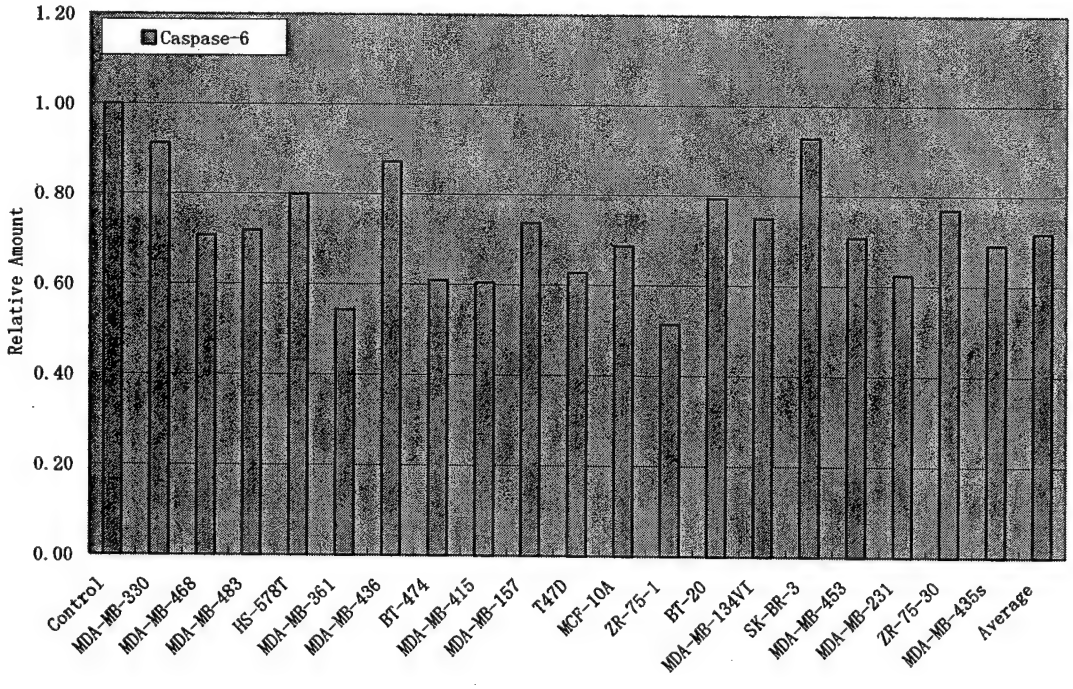
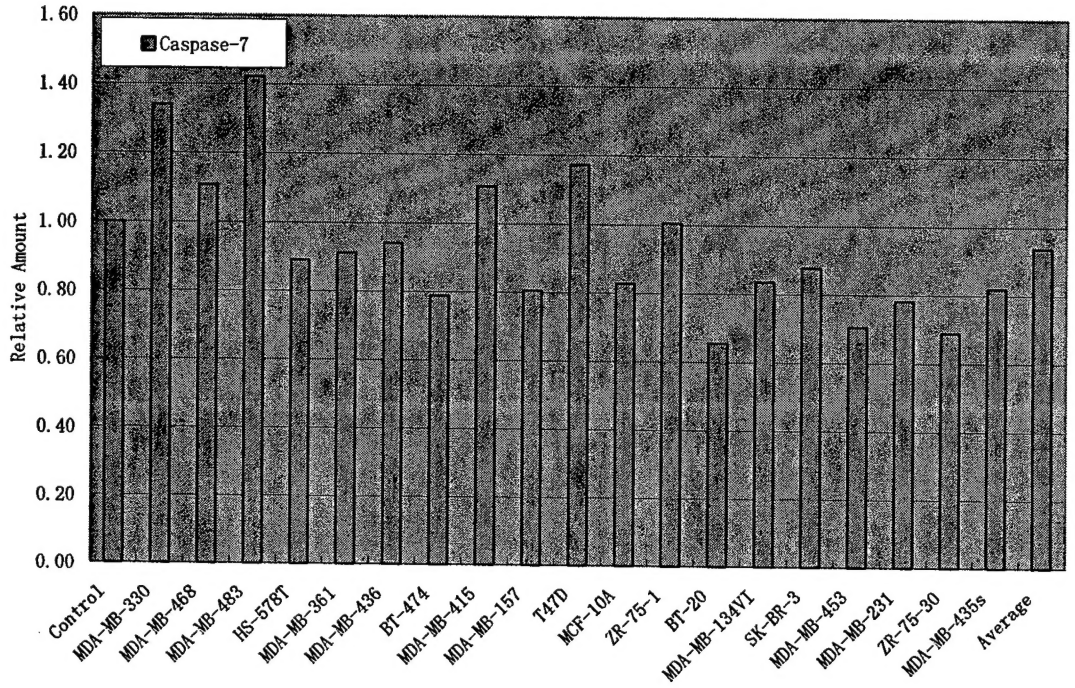
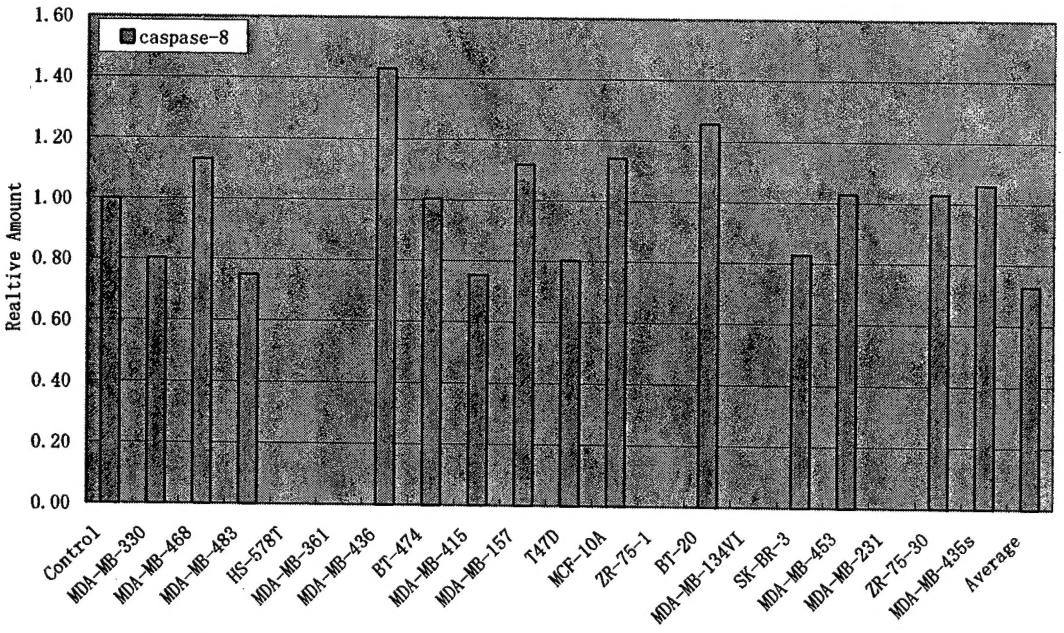
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


Caspase-6
Relative Amount
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


Caspase-7
Relative Amount
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


caspase-8
Realtive Amount
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
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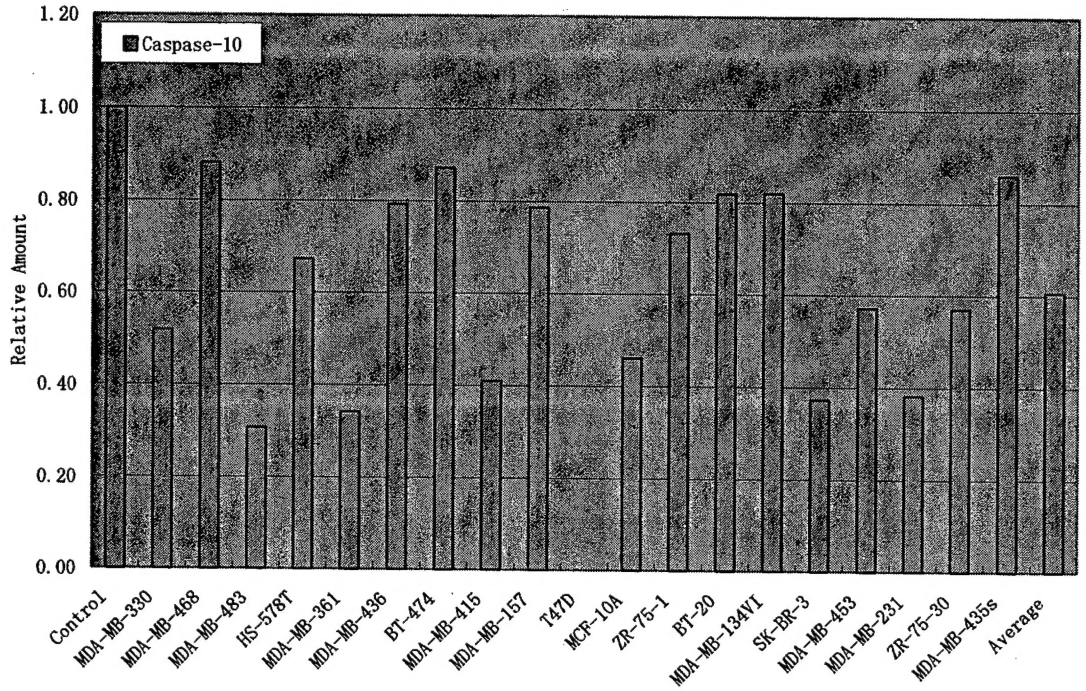
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average


Caspase-10
Relative Amount
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average

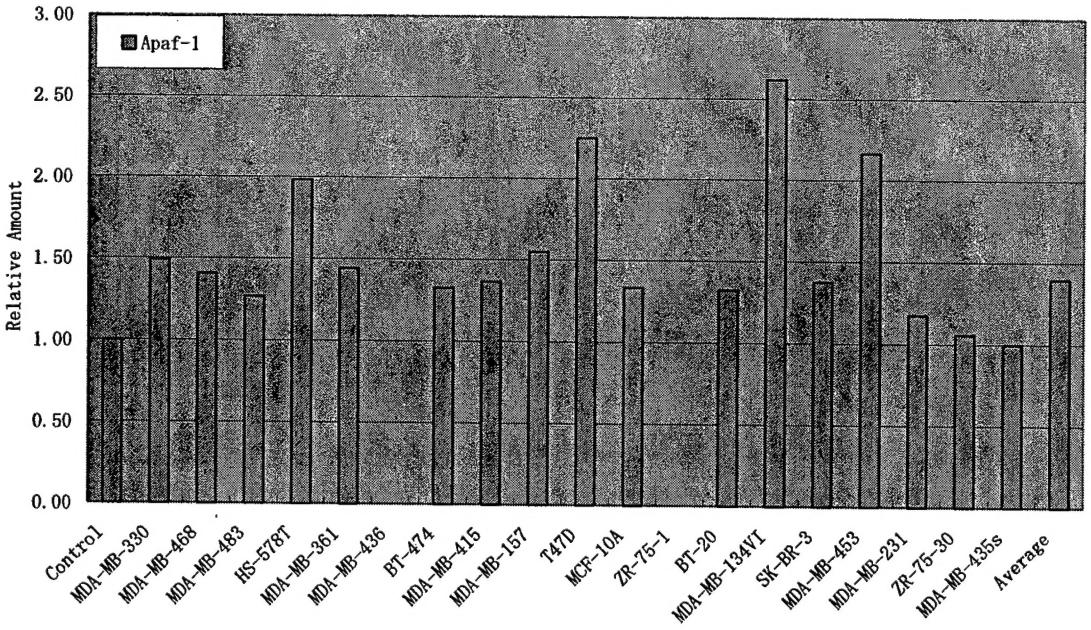
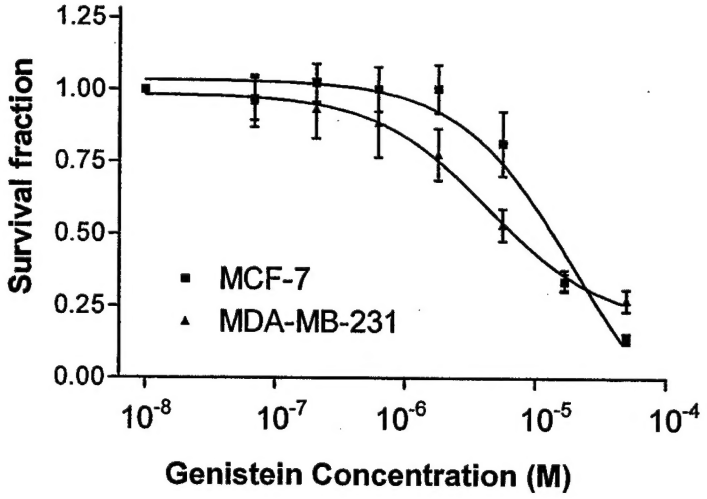

Apaf-1
Relative Amount
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Control
MDA-MB-330
MDA-MB-468
MDA-MB-483
HS-578T
MDA-MB-361
MDA-MB-436
BT-474
MDA-MB-415
MDA-MB-157
T47D
MCF-10A
ZR-75-1
BT-20
MDA-MB-134VI
SK-BR-3
MDA-MB-453
MDA-MB-231
ZR-75-30
MDA-MB-435s
Average

Figure 4. Growth inhibition of genistein on MCF-7 and MDA-MB-231 breast cancer cells. Each cell line was treated the indicated concentration of genistein for 6 days. Survival fractions were measured using MTT assay. The EC_{50} was calculated based on three separate experiments.



EC_{50} for MCF-7 cells:	1.93e-5 M (19.3 μM)
EC_{50} for MDA-MD-231 cells:	4.36e-6 M (4.36 μM)

Fig. 5. Expression of caspases in MDA-MB-231, MCF-7, MCF-7/PV and MCF-7/C cells. Caspase-3 levels were detected using Western blot. MCF-7/pv and MCF-7/C were the cells transfected with pBabepuro vector and pBabepuro-caspase-3 plasmid respectively.

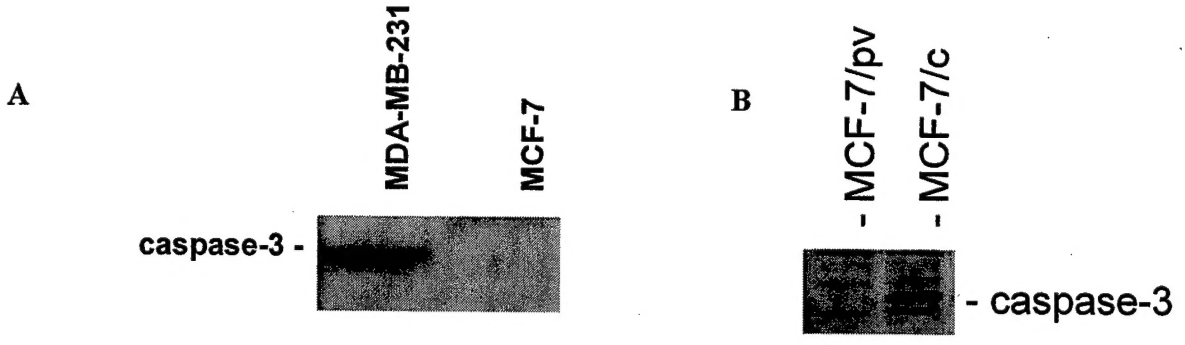


Fig. 6. Reconstitution of caspase-3 sensitizes MCF-7 cells to genistein. MCF-7/pv and MCF-7/C cells treated the indicated concentration of genistein for 6 days. Survival fractions were measured using MTT assay..

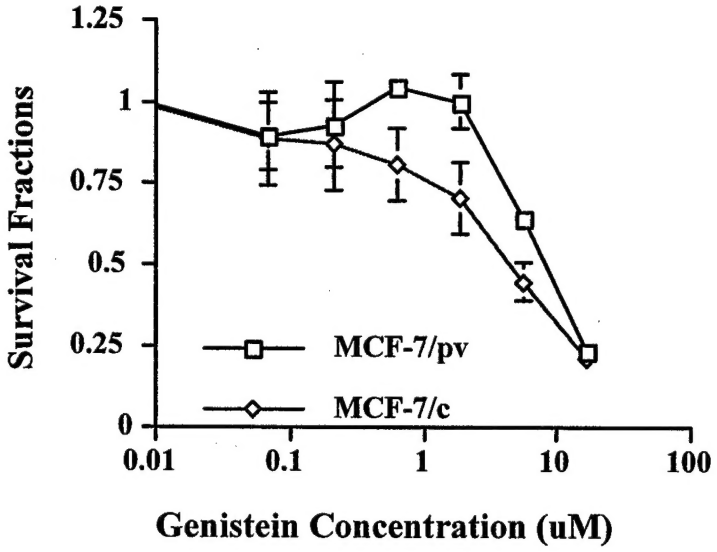


Fig. 7. Cleavage of PARP in genistein treated MCF-7/pv and MCF-7/C cells. Both cell lines were treated with genistein at indicated concentration for 48 hrs. Total (p116) and cleaved (p89) PARP were detected using Western blot.